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Sampling the Light-Organ Microenvironment of *Euprymna scolopes:* Description of a Population of Host Cells in Association With the Bacterial Symbiont *Vibrio fischeri*

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Abstract. The symbiosis between the squid *Euprymna scolopes* and the luminous bacterium *Vibrio fischeri* has a pronounced diel rhythm, one component of which is the venting of the contents of the light organ into the surrounding seawater each day at dawn. In this study, we explored the use of this behavior to sample the microenvironment of the light-organ crypts. Intact crypt contents, which emerge from the lateral pores of the organ as a thick paste-like exudate, were collected from anesthetized host animals that had been exposed to a light cue. Microscopy revealed that the expelled material is composed of a conspicuous population of host cells in association with the bacterial symbionts, all of which are embedded in a dense acellular matrix that strongly resembles the bacteria-based biofilms described in other systems. Assays of the viability of expelled crypt cells revealed no dead bacterial symbionts and a mixture of live and dead host cells. Analyses of the ultrastructure, biochemistry, and phagocytic activity of a subset of the host cell population suggested that some of these cells are macrophage-like molluscan hemocytes.

Introduction

The microenvironment surrounding extracellular bacterial symbionts that associate with animal hosts is the dynamic zone of interchange between the partners. In a number of associations, most notably the cow rumen (Flint, 1997) and the termite hindgut (Breznak, 1982; Breznak and Brune, 1994), the characterization of this interface has provided insight into the basic nature of the symbiosis, including aspects of nutrient exchange, host immune response, and the control of symbiont number. In such consortial associations, the contribution of any given symbiont species to the dynamics of the whole has been difficult to assess. Thus, the precise mechanisms by which the environment is created and maintained have not been determined.

The symbiosis between the Hawaiian bobtail squid *Euprymna scolopes* and its bioluminescent bacterial partner *Vibrio fischeri* provides a research system complementary to the more prevalent consortial symbioses (Ruby, 1996; McFall-Ngai and Ruby, 1998). Because both partners are culturable outside of the symbiosis, and the bacterial symbiont can be genetically manipulated, this two-species association can be used to study the underlying biochemical and molecular contributions of each partner to the dynamics of the symbiosis.

The microenvironment surrounding the bacterial symbionts in this symbiosis can now be readily analyzed (Graf and Ruby, 1998). The squid houses its extracellular symbionts in the epithelial crypts of a conspicuous bilobed light organ located in the center of its mantle cavity (McFall-Ngai and Montgomery, 1990). Lee and Ruby (1994) and Boettcher *et al.* (1996) showed that the symbiosis is characterized by a pronounced diel rhythm, one aspect of which is the daily venting of 90% of the bacterial culture into the surrounding seawater. At dawn, each day, the animal expels the bacteria-containing crypt material into the mantle cavity through lateral pores on either side of the light organ (see Fig. 1). This daily venting appears to have a variety of consequences for the symbiosis. Lee

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and Ruby (1994) provided evidence that, in addition to the obvious function of controlling symbiont number, venting increases population densities of *V. fischeri* in the ambient seawater, which is essential for the horizontal transmission of the symbiosis between generations; specifically, they showed that the natural seawater is only infective to newly hatched squids when it is sampled from environments with large populations of adults. Under laboratory conditions, Graf and Ruby (1998) exploited this diel venting behavior of the host to show that the squid host provides the bacteria in the symbiosis with amino acids.

In the present study, we explore the cellular components of the vented crypt contents. Our data show that the host vents a dense assemblage of its own cells in association with the bacterial symbionts. This mixed population of cells is embedded in a conspicuous acellular matrix. Among the host cell population, there is a subset of cells that have a morphology, biochemistry, and phagocytic activity suggestive of macrophage-like mollusean hemocytes. In addition, we demonstrate that the animals can be experimentally induced to vent their crypt contents at times of day other than dawn, a feature that will provide an opportunity for future studies of diel fluctuations in the microenvironment of the light organ crypts.

Materials and Methods

General procedures

Adult *E. scolopes* were collected with the use of dip nets from shallow, subtidal regions surrounding Oahu, Hawaii, and were either transported to the University of Southern California, Los Angeles, and maintained in a 265-liter recirculating aquarium at 23°C, or maintained in flow-through seawater aquaria at Kewalo Marine Laboratory, University of Hawaii, Manoa.

All chemicals were obtained from Sigma Chemical Co. (St. Louis, Missouri) unless otherwise stated. Fixatives, embedding media, and supplies for electron microscopy were purchased from Ted Pella, Inc. (Redding, California).

Characterizing natural venting behavior and obtaining crypt contents

We used the methods of Graf and Ruby (1998) to induce venting behavior and to acquire the contents of the light organ crypts for further analysis. Briefly, adult animals were maintained under natural environmental light conditions of about 12 h of light and 12 h of darkness. Minutes prior to dawn, just before venting normally occurs (Lee and Ruby, 1994), animals were anesthetized in 2% ethanol in seawater. Under red light, we made a midventral transection of the mantle to expose the light organ. The animals were then subjected to a constant light stimulus, using a 150-W halogen light source placed several centimeters above the eyes. Either immediately or within 60 min of the onset of the stimulus, they vented their light organ contents. This variation in the timing of venting following the stimulus did not affect the factors that were characterized in this study. As they were vented, the crypt contents were collected with a 5-μl hematocrit tube fitted with a plunger. To determine whether light, and not another aspect of our procedure (*i.e.*, anesthesia or dissection), was the necessary stimulus for the induction of venting, animals were anesthetized during their natural dark period and dissected, but not given a light stimulus.

The possibility that venting behavior occurs in newly hatched squid at the first dawn following hatching was tested as follows. Juvenile squid were anesthetized for 60 s in a solution containing 0.37 *M* $MgCl_2$ and seawater (1:1). Acridine orange was added to the solution to a final concentration of 5 ng/ml, which uniformly stained the host cells. The light organs were exposed by removing the mantle tissue, animals were exposed to a light stimulus, and the vented contents were viewed under confocal microscopy.

Preparation for electron microscopy

For scanning electron microscopy (SEM), the exuded crypt contents were placed on a nitrocellulose membrane, which was then immersed and fixed for 15 min in a solution of 5% formalin in filtered seawater (FSW). The samples were rinsed in FSW, dehydrated in a 15%–100% ethanol series, desiccated with hexamethyldisilazane, sputter coated with gold, and examined with a Cambridge 360 scanning electron microscope.

For transmission electron microscopy (TEM), the exudate was fixed in 4% glutaraldehyde in 0.1% sodium cacodylate with 0.45 *M* NaCl, pH 7.4 (Fixative A), for 30 min. Samples were rinsed for 15 min in 0.1% sodium cacodylate with 0.45 *M* NaCl, pH 7.4 (Buffer A) and then postfixed in 1% osmium tetroxide in Buffer A for 20 min followed by rinsing with Buffer A for 10 min. Samples were then dehydrated through a graded series from 30% to 100% ethanol in distilled water followed by infiltration with propylene oxide. Samples were placed in a 50:50 mixture of propylene oxide and unaccelerated Spurr (Spurr, 1969) for 15 min, and then transferred first to 100% unaccelerated Spurr for 3 h, and then 100% accelerated Spurr for 2 h. Samples were embedded in freshly prepared accelerated Spurr at 67°C for 48 h. The embedded exudate was sectioned (80 to 90 nm thick), stained with Reynolds lead citrate solution and 3% uranyl acetate, and viewed with a JEOL CX-100 transmission electron microscope.

To determine whether the composition of vented mate-

rial was influenced by the experimental procedure by which it was obtained (*i.e.*, from anesthetized, ventrally dissected animals), we analyzed the crypt contents in intact, unanesthetized animals just prior to venting. Whole adult animals were placed at dawn directly in Fixative A. The light organs were then dissected out and prepared for TEM as described previously (McFall-Ngai and Montgomery, 1990).

Analysis of animal and bacterial cells in the exudate

The total numbers of bacterial and animal cells present in fresh exudate were determined for adult specimens [n = 3; average mantle length, 25 mm]. To quantify culturable bacterial cells per microliter in the exudate, serial dilutions of fresh exudate were plated on seawater tryptone (SWT) agar medium and allowed to incubate at 25°C overnight (Ruby and Asato, 1993). The resulting colonies were counted, giving the number of colony forming units (CFUs) in the exudate material. This plating technique detects culturable bacterial cells with greater than 95% efficiency (Ruby and Asato, 1993). *In situ* viability of bacterial cells in the exudate was determined by fluorescence viability staining (*Bac*Light Live/Dead Viability Assay Kit, Molecular Probes, Eugene, OR). We used two nucleic acid stains, which are provided separately and mixed immediately prior to application: SYTO-9, which is taken up by all cells and fluoresces green, and propidium iodide, which fluoresces red and is only taken up by dead or dying bacterial cells with damaged membranes (Lloyd and Hayes, 1995). Cells were observed by confocal microscopy immediately after collection, and then 2 h later to ensure that propidium iodide had penetrated dead *V. fischeri*.

The number of animal cells was determined as follows. Fresh exudate of a known volume was placed in a Petroff-Hauser hemacytometer, and the cells were either counted under phase contrast microscopy or stained with acridine orange and counted under fluorescent microscopy. Viability of animal cells in the exudate was determined by exposing the material to trypan blue, which is excluded from healthy animal cells but taken up freely by dead and dying cells with compromised membranes. Samples were exposed to 0.1% trypan blue in FSW for 3.0 min, rinsed in FSW, and examined under light microscopy.

To determine the abundance of acidic compartments (*e.g.*, lysosomes) within the animal cells, fresh exudate was exposed to a 1 μM solution of LysoTracker Green (Molecular Probes, Eugene, OR) for 30 min, rinsed in FSW, and viewed under confocal microscopy. LysoTracker consists of a fluorophore moiety linked to a weak base that permeates cell membranes; it concentrates in cellular compartments with low internal pH, such as lysosomes, where it fluoresces under acidic conditions (Diwu *et al.*, 1994).

Experimental manipulation of venting behavior

To determine whether crypt contents could be obtained at times other than dawn, we attempted to induce venting behavior by exposing *E. scolopes* adults to our experimental conditions (*i.e.*, anesthetic, ventral dissection, and a light stimulus) at different times during the day. Specifically, we assayed in the hours preceding [−12 h, −9 h, −6 h, −4 h, and −1 h] and following [+4 h, +6 h, +8 h, and +10 h] dawn, when the light organ would presumably have either abundant or depleted crypt contents, respectively. In addition, to determine whether the crypt contents would be retained past dawn in the absence of the natural light cue, adult animals were maintained in the dark for up to 8 h after their natural venting time and then exposed to our experimental conditions.

Results

Natural venting behavior

After a continuous light stimulus of 5–60 min, the release of the crypt contents (Fig. 1) was preceded by a large contraction of the light organ. The exudate that resulted from this event emerged from the pores of the light organ as a thick, white, paste-like material (Fig. 1B). An adult animal with a mantle length of 25 mm typically vented between 10 and 20 μl of exudate material, which was then collected intact for further analyses. Animals that were anesthetized and ventrally dissected, but not exposed to a light stimulus, did not expel their crypt contents—neither during the natural dark cycle nor after dark adaptation. Therefore, a light stimulus is required to induce venting, and the experimental conditions themselves did not cause this behavior.

Under the experimental conditions used for adults, juvenile animals also exhibited venting behavior beginning with the first dawn after hatching. Large eukaryotic cells of a diameter similar to those seen in adults (about 10 μm) were observed emerging from the pores leading to the juvenile crypts (Fig. 1C).

Cellular constituents of the exudate

Observations of exudate under light microscopy revealed a mixture of animal and bacterial cells. SEM of the exudate showed that the population of animal and bacterial cells is surrounded by a dense matrix (Fig. 2A–C). Animal cells in the exudate were 10 to 20 μm in diameter. Their membranes often had a ruffled appearance, and cytoplasmic blebbing was regularly observed, but these cells otherwise appeared healthy (Fig. 2D). Both SEM and TEM revealed a close association between the animal cells and bacterial cells within the exudate, with bacterial cells often adhering to the surface of the eukaryotic cells (Fig. 2C–D). TEM of the exudate matrix

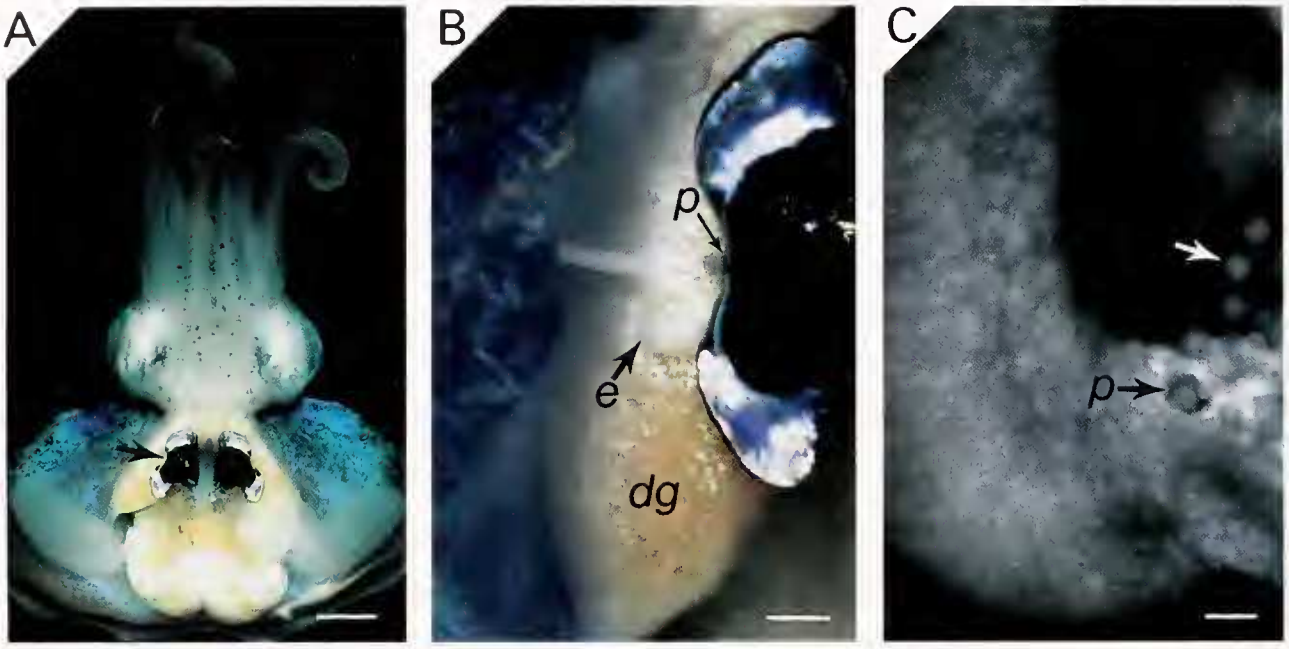

Figure 1. Venting of crypt contents by *Euprymna scolopes*. (A) Ventral dissection of an adult, revealing the bilobed light organ (arrow). Scale, 5 mm. (B) Exudate emerging from one of the lateral pores of the light organ. The exudate can be seen lying across the digestive gland (yellow), which is directly dorsal to the light organ. Scale, 1 mm. (C) Animal cells (white arrow) emerging from the pore of a 48-h juvenile squid. Scale, 25 μm. p, pore; e, exudate; dg, digestive gland.

showed a mixture of particulate material suggestive of cell membranes, possibly of lysed host cells (Fig. 2D).

Based on counts of CFUs, an average of 2.8×10^9 ($\pm 1.3 \times 10^9$, $n = 3$) bacterial cells were contained within the exudate of an *E. scolopes* adult. After venting, homogenates of the bacteria-containing crypt epithelia of the light organ of these same animals contained, on average, 10^8 bacterial cells; thus, 5% to 10% of the symbiont population remained in the hosts. This number of bacteria corresponds to estimates of symbiont retention in animals that were allowed to vent naturally (Lee and Ruby, 1994). In adult squid (average mantle length, 25 mm), the number of animal cells in the exudate, as determined by light and fluorescence microscopy, averaged between 10^3 and 10^4 total cells.

Control studies with fixed, intact, unvented light organs showed animal and bacterial cells in the collection ducts leading up to and in the crypt spaces (Fig. 3). These cells were similar in appearance to cells found in freshly vented exudate samples. TEM revealed that the accompanying matrix material was also similar to that observed in exudate (data not shown).

Viability of animal and bacterial cells

About half the freshly vented animal cells that were stained with trypan blue were observed to exclude the dye, indicating that they were viable. The remainder took up the blue stain, indicating that they were dead or dying cells with compromised membranes (Fig. 4A). Staining patterns under confocal microscopy, with two nucleic acid stains that distinguish live and dead bacterial cells, indicated that the vast majority of bacteria in the exudate are viable (Fig. 4B). To ensure that these nucleic acid stains could reveal dead cells, the crypt contents were stained 2 h after being expelled. An increase in bacterial cells fluorescing red was observed 2 h after venting, so the propidium iodide revealed dying or dead *V. fischeri* (data not shown).

Acidic compartments of animal cells in the exudate

The animal cells in the exudate were examined for the presence of acidic compartments characteristic of phagocytic or macrophage-like cells. To this end, fresh exudate stained with LysoTracker dye was observed with both light and fluorescence microscopy. Under differential interference contrast (DIC) microscopy, exudate cells appeared to be surrounded by matrix and adherent bacterial cells (Fig. 5A). In the presence of LysoTracker, fluorescent compartments were seen within the animal cells, whereas the nucleus and surrounding extracellular matrix, as well as bacterial cells in the mixture, showed no fluorescence (Fig. 5B).

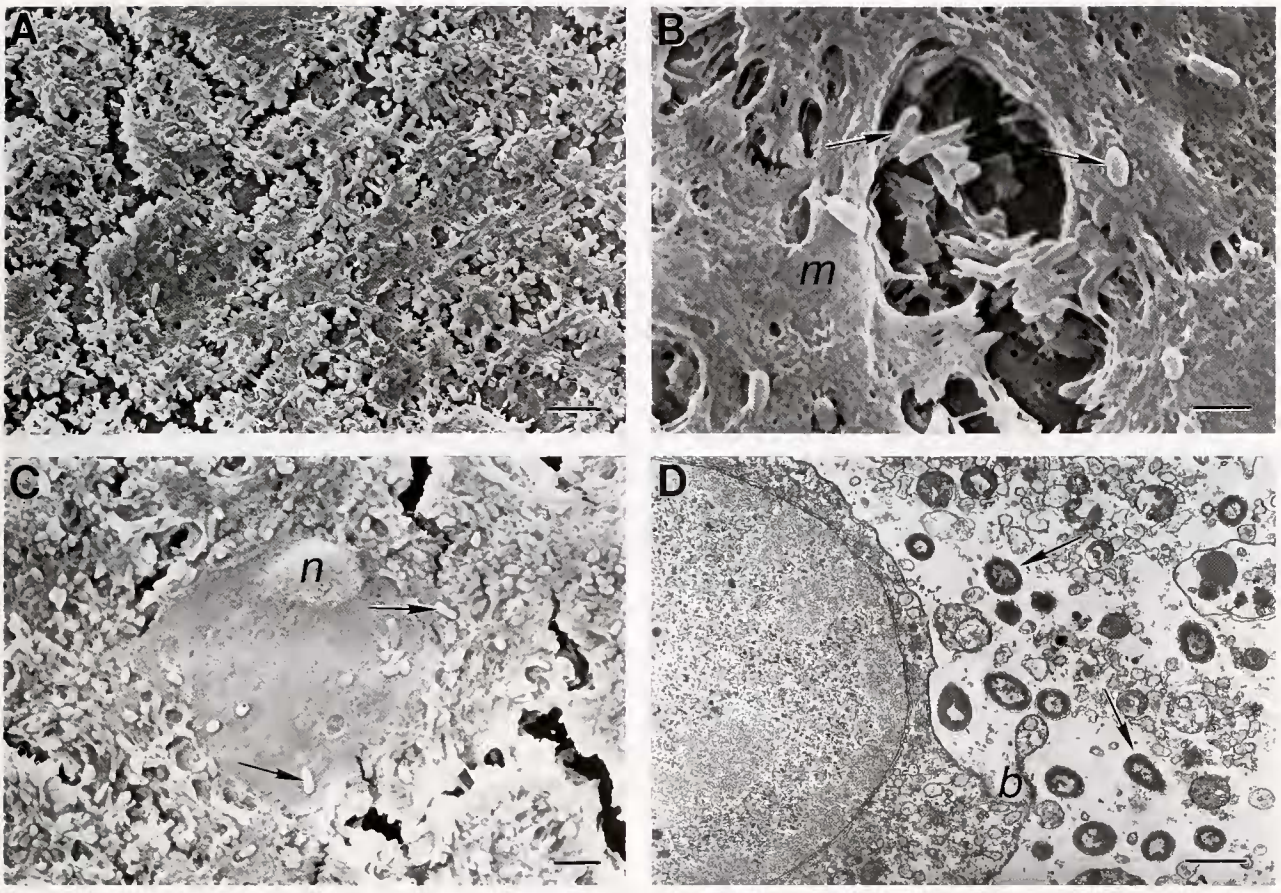

Figure 2. Electron micrographs of freshly collected exudate (A–C were taken with a scanning scope, and D with a transmission scope). (A) Bacterial cells embedded in a thick matrix. Scale, 3 μm. (B) Higher magnification, showing individual bacteria (arrows) surrounded by the matrix. Scale, 1 μm. (C) The outline of animal cells surrounded by bacteria (arrows). Scale, 2 μm. (D) Bacterial cells (arrows) adjacent to a larger animal cell. The membrane exhibits ruffling, and cytoplasmic blebbing is evident. The bacteria are surrounded by a mixture of particulate matter that constitutes the matrix. Scale, 1 μm. m, matrix; n, nucleus; b, cytoplasmic blebbing of the host cell.

Evidence of bacterial phagocytosis in light organ cells

Eukaryotic cells in the crypt spaces of intact light organs occasionally contained intracellular bacteria (Fig. 6). Like the majority of the animal cells within the exudate, these cells averaged 10 μm in diameter and often contained 3–5 bacterial cells each. The bacteria appeared to be contained within membrane-bound vacuoles of the animal cells, and many of these bacterial cells appeared to be undergoing degradation.

Manipulation of venting behavior

Under our experimental conditions, we were able to induce venting behavior between 1 and 12 h prior to dawn, *i.e.*, during the dark portion of the animal's natural cycle—the time period when the bacterial population of the light organ is at its highest. This behavior could not be induced, however, during the light portion of the animal's natural cycle; this is the period between 4 and 10 h after dawn, when the crypt spaces should have the lowest amount of extracellular constituents, *i.e.*, matrix material, as well as animal and bacterial cells.

Animals kept in the dark past dawn retained their crypt contents until given a light stimulus. Under these conditions, we could induce expulsion up to 8 h past the natural venting time. These data suggest that the behavior is not under an independent circadian rhythm, but requires a light cue each day.

Discussion

The opportunity to sample the microenvironment surrounding bacterial symbionts is rare because, in most associations, the bacteria are embedded deeply within tissues that are difficult to access. In the present study, taking

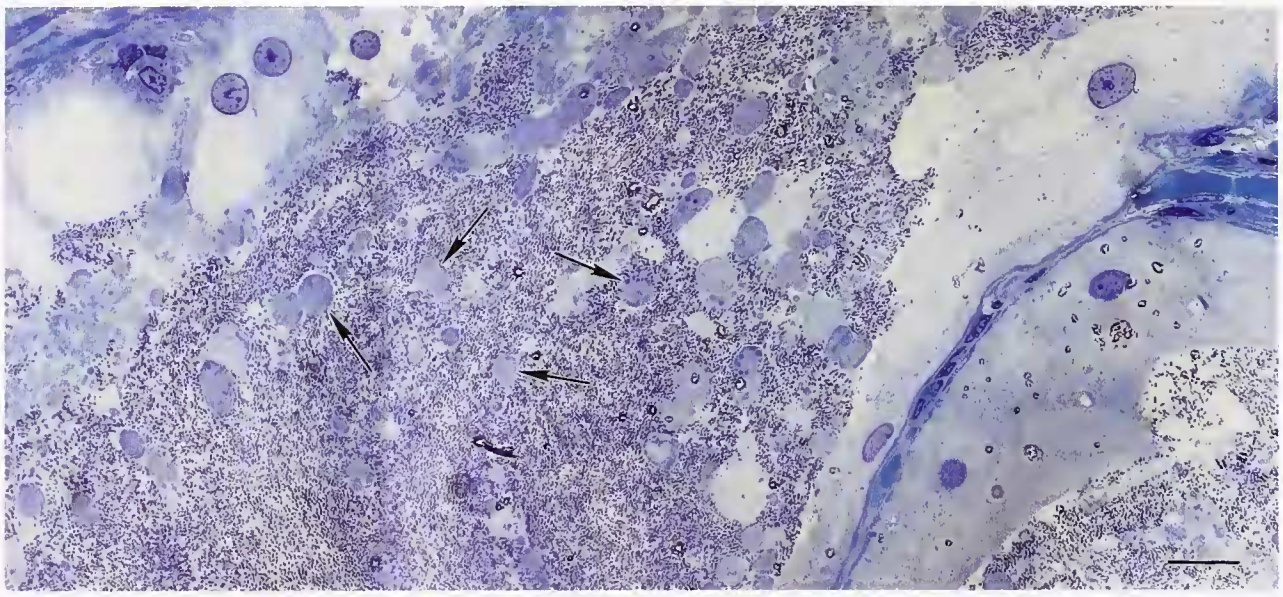

Figure 3. Exudate in the duct and anterior crypt spaces of a sectioned adult light organ. The image is a montage of light micrographs of tissue that was fixed at dawn, just before the natural venting. The typical experimental conditions that produce venting (*e.g.*, anesthetic and light stimulus) were not applied. Eukaryotic cells (arrows) in the crypt and duct spaces are surrounded by bacterial cells. The sample was stained with 2% toluidine blue. Scale, 20 μm.

advantage of a natural behavior of the host squid, we have described the cellular components of the microenvironment of the symbiotic light organ and have determined the feasibility of sampling this microenvironment under experimental conditions.

These studies have revealed that (1) the diel venting

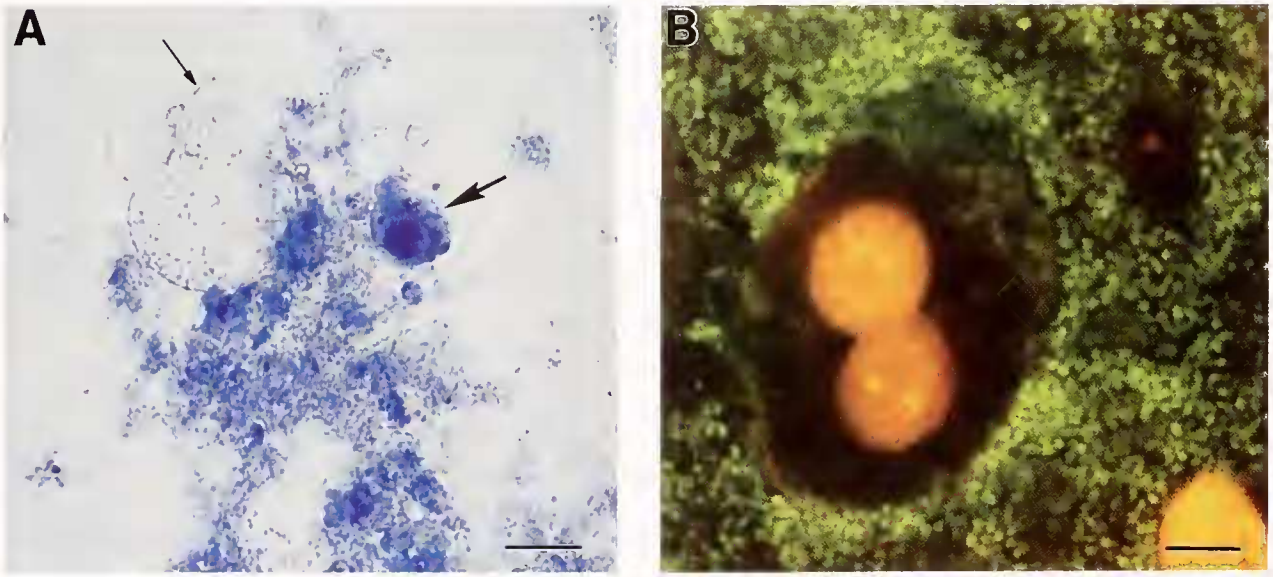

Figure 4. Viability of cells in the exudate. (A) Results of staining the exudate with 0.1% trypan blue to test viability of animal cells. The exudate contains a mixture of living cells, which exclude the stain and remain a golden hue (small arrow), and dead or dying cells, which do not exclude the stain (large arrow). The matrix also interacted with the dye and appears blue. Magnification, 400 ×. Scale, 10 μm. (B) Results of two nucleic acid fluorescent stains that distinguish viable from dead bacterial cells. The vast majority of freshly emerged bacterial cells were viable, as indicated by the green field of bacteria surrounding the animal cell. Scale, 5 μm.

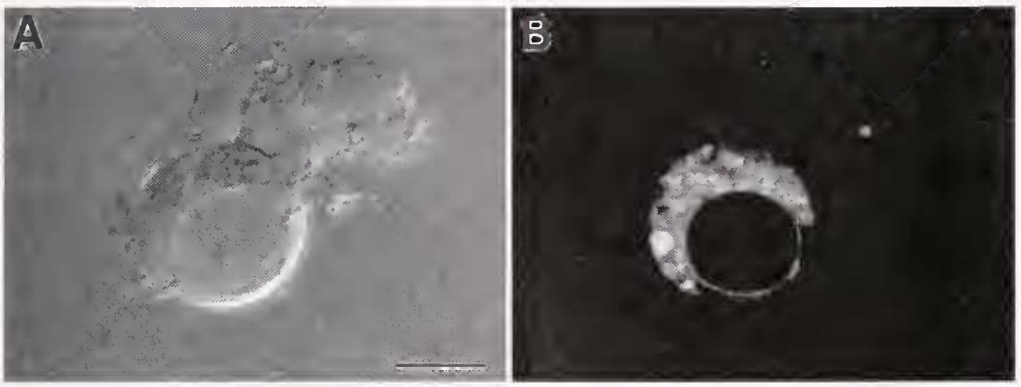

Figure 5. Stained acidic compartments within a freshly vented animal cell. (A) Differential interference microscopy; the cell is surrounded by bacteria and matrix material. Scale, 10 μm. (B) The same cell stained with 1 μM LysoTracker; note the acidic compartments, possibly lysosomes. Scale, 10 μm.

of the symbiotic organ contents by the host squid provides an intact sample of the light organ crypt microenvironment; (2) this diel behavior, which occurs in juveniles as well as adults, is dependent upon a light cue; (3) the cellular constituents of the crypts consist of a population of viable *V. fischeri* cells in association with a mixture of live and dead host cells; (4) the characteristics of some of the animal cells found in the exudate suggest that these cells are molluscan phagocytic hemocytes (Cheng, 1981; Sminia, 1981; Cowden and Curtis, 1981); (5) these host and bacterial cells are embedded in a conspicuous matrix similar in appearance to other bacteria-based biofilms; and (6) the venting of crypt contents can be experimentally induced at times in the diel cycle other than dawn.

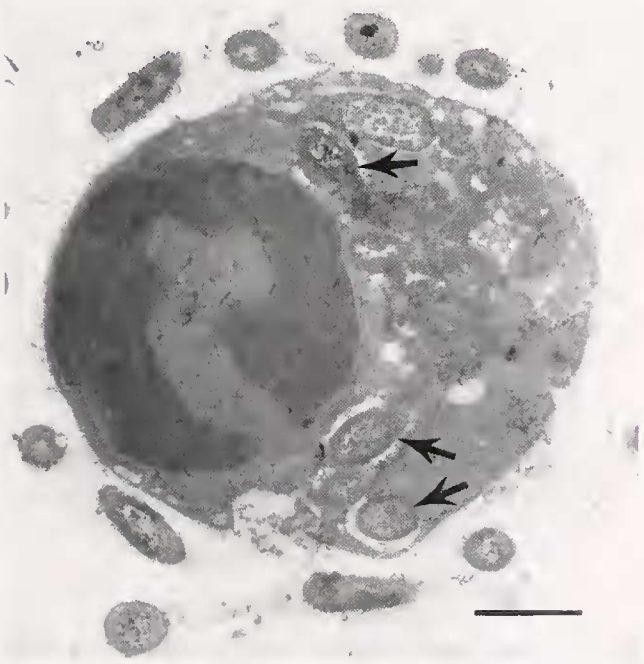

Figure 6. Bacteria within a crypt cell. A TEM of a eukaryotic cell found within the crypt spaces of a juvenile *E. scolopes* light organ. This cell contains several intracellular bacteria (arrows). Scale, 1 μm.

Our finding that about 90% of the symbiont culture is vented suggests strongly that this behavior is controlling the number of symbionts in the light organ (Lee and Ruby, 1994; present study). Research on symbiotic associations between animals and their bacterial partners has demonstrated that symbiont population density is regulated in a variety of ways. These control mechanisms can be grouped into two broad categories that work either alone or in concert: control of bacterial growth rate and elimination of excess bacterial cells. In the former mode, the host environment, while sustaining the viability of the symbionts, presents a biochemical milieu that attenuates bacterial cell division. In the latter, elimination of supernumerary symbionts is typically accomplished by the regular venting of the bacterial cells from the host tissues, the digestion by the host of the bacterial cells, or both. Digestion can be either extracellular or intracellular, the latter typically following the engulfment of the bacterial cell by a phagocytic host cell. Previous research and the results presented here provide evidence for a multifaceted control of the symbiont population in the squid-vibrio symbiosis. Lee and Ruby (1994) demonstrated that the bacterial growth rate in the adult light organ (average doubling time of approximately 4.8 h) is suppressed in comparison with the physiological potential for growth seen under culture conditions (average doubling time of 0.5 h; Ruby and Asato, 1993). However, the former growth rate is still sufficiently high that, although the numbers of bacteria are set back at dawn to a lower population density by the venting behavior, the light organ will be repopulated with symbionts over the course of the day. Slow growth rates, coupled with shedding of symbionts into the environment, have been documented previously in symbioses between animal hosts and their

luminous bacterial partners, although no rhythm or dependency on cues was noted (Haygood *et al.*, 1984).

In addition to the suppression of growth rate and the diel venting of symbionts, the phagocytosis of bacterial cells by host macrophage-like cells that we observed in this study (Fig. 6) may represent yet another level of control of the symbionts in these associations. Tebo *et al.* (1979) showed bacteria in host cells in the light organ of the monocentrid fish *Monocentris japonicus*, whose light organ contains the symbiont *V. fischeri*. However, those bacterial cells were in the epithelium rather than in the macrophage-like cells seen in the light organ of *E. scolopes*. At this point, neither they nor we have determined whether the bacteria within host cells are *V. fischeri* or other bacterial species that are being eliminated to maintain *V. fischeri* as the sole symbiont; *i.e.*, we do not know whether the macrophage-like host cells in the squid light organ function principally in control of *V. fischeri* symbiont number or in maintenance of light organ specificity, or both. Thus, the determination of the precise function of these cells in the dynamics of this symbiosis awaits their further characterization. However, to our knowledge, this report represents the first documentation of macrophage-like cells in direct association with the symbionts in a light organ.

Whatever the function of these host macrophage-like cells in the crypt spaces, their association with the population of bacterial symbionts is not unexpected. In molluscs, macrophage-like hemocytes are reported to be involved in phagocytosis and digestion of pathogens in many tissues and their associated lumina (Cheng, 1981; Sminia, 1981; Cowden and Curtis, 1981). Vertebrate macrophages, which are thought to be derived evolutionarily from this invertebrate cell type (Ottaviani and Franceschi, 1997), are also found in similar circumstances. For example, mononuclear phagocytes are released into the blood stream and spread to all tissues including epithelia (Stewart *et al.*, 1994), and lymphocytes are a common constituent of the cellular community that lines the mammalian intestine (James and Zeitz, 1994).

The other animal cells in the crypt environment that do not resemble healthy, functioning macrophage-like molluscan hemocytes may be dead or dying hemocytes, or they may be epithelial cells shed from the lining of the crypt spaces. The shedding of epithelial cells in response to interaction with bacteria or bacterial by-products is known to occur in other instances. For example, bacterial lipopolysaccharide will induce shedding of murine uroepithelial cells, which is believed to be a mechanism by which the host evades pathogenic bacteria (Aronson *et al.*, 1988). The exact definition of the host cell types in the crypt space and the origin of these cells, thus, also awaits future studies.

All of the bacterial and animal cells contained in the exudate are suspended in a thick, heterogeneous matrix. SEM showed that this matrix resembles other bacteria-associated biofilms, including those associated either with inorganic substrates or animal tissues (Pearl, 1985). TEM revealed a complex mixture of membranes and particulate matter. Matrix ultrastructure of the squid light organ appears similar to that described in the light organs of the macrourid fish *Caelorhincus* (W. Loh, University of Sydney, pers. comm.). The exact nature of the constituents, as well as the relative contributions of the animal host and the bacterial symbionts to the matrix material, remain to be determined. To date, only one constituent has been defined, *i.e.*, the host-derived amino acids that supply the growing symbiont population with these metabolic building blocks (Graf and Ruby, 1998).

The finding that venting behavior can be induced experimentally during the 12 h preceding the natural expulsion event at dawn paves the way for examination of the microenvironment of the crypts during this portion of the diel cycle of the symbiosis. Boettcher *et al.* (1996), in studies of the diel rhythm of the light organ, showed that this 12-h period contains the highest and lowest per cell luminescence of *V. fischeri*. They interpreted this finding to suggest that significant fluctuations in the biochemistry and physiology of the crypt environment occur between the hours around dusk and the hours around dawn. After venting, the remaining 10% of the symbiont population divides and repopulates the light organ, so that by dusk, there is once again a full complement of bacteria in the crypts. Whether the animal delivers nutrients steadily over the period of about 12 h following venting, sustaining a slow bacterial growth rate over this period, or whether it maintains a low bacterial population until the late afternoon, when it delivers a large pulse of nutrients that stimulates rapid bacterial growth, remains to be determined. Defining changes in the biochemistry of the microenvironment during the day may provide insight into the mechanisms by which luminescence is controlled. In addition, such analyses will define other types of metabolic and regulatory changes that accompany the diel rhythm of this symbiosis. At present, we cannot experimentally induce venting of the crypt contents during the light portion of the animal's cycle. Thus, a full understanding of the dynamics of symbiont population growth in the light organ over the entire day awaits refinement of our current method or reliance on alternative methods of sampling this environment.

The accessibility of the bacterial microenvironment in this symbiosis makes a rich frontier available for the study of the dynamics of an animal-bacterial association. Future studies will focus on defining both the biochemical constituents of the matrix material and the nature of the animal cells that occur among the bacterial symbionts.

Acknowledgments

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